



Cronobacter sakazakii in foods and factors affecting its survival, growth, and inactivation

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ABSTRACT

Cronobacter sakazakii has been isolated from a wide range of environmental sources and from several foods of animal and plant origin. While infections caused by *C. sakazakii* have predominantly involved neonates and infants, its presence on or in foods other than powdered infant formula raises concern about the safety risks these foods pose to immunocompromised consumers. We have done a series of studies to better understand the survival and growth characteristics of *C. sakazakii* in infant formula, infant cereal, fresh-cut produce, and juices made from fresh produce. Over a 12-month storage period, the pathogen survived better in dried formula and cereal at low a_w (0.25–0.30) than at high a_w (0.69–0.82) and at 4 °C compared to 30 °C. *C. sakazakii* grows in formulas and cereals reconstituted with water or milk and held at 12–30 °C. The composition of formulas or cereals does not markedly affect the rate of growth. *C. sakazakii* grows well on fresh-cut apple, cantaloupe, watermelon, cabbage, carrot, cucumber, lettuce, and tomato at 25 °C and in some types of produce at 12 °C. Treatment of fresh fruits and vegetables with sanitizers such as chlorine, chlorine dioxide, and a peroxyacetic acid-based solution causes reductions of 1.6–5.4 log CFU/apple, tomato, and lettuce. Cells of *C. sakazakii* in biofilms formed on stainless steel and enteral feeding tubes or dried on the surface of stainless steel have increased resistance to disinfectants. Death of cells in biofilms is affected by atmospheric relative humidity. These studies have contributed to a better understanding of the behavior of *C. sakazakii* in and on foods and on food-contact surfaces, thereby enabling the development of more effective strategies and interventions for its control.

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1. Introduction

Neonatal infections believed to have been caused by *Cronobacter sakazakii*, formerly *Enterobacter sakazakii* (Iversen et al., 2008), were first reported by Urmenyi and Franklin (1961). Numerous cases have been subsequently described. A book (Farber and Forsythe, 2008) and several reviews (Nazarowec-White and Farber, 1997a; Lai, 2001; Iversen and Forsythe, 2003; Lehner and Stephan, 2004; Gurtler et al., 2005; Bowen and Branden, 2006; Drudy et al., 2006; Mullane et al., 2006; Friedemann, 2007) have summarized information on taxonomy, biochemical characteristics, epidemiology, pathogenicity, clinical etiology, and survival and inactivation characteristics of *C. sakazakii* in foods and the environment.

Reconstituted powdered infant formula and powdered milk have been the most common vehicles implicated in neonatal *C. sakazakii* infections. Other unidentified sources of the pathogen were involved in cases of infections in infants, children, and immunocompromised

adults having underlying medical conditions (Jimenez and Gimenez, 1982; Pribyl et al., 1985; Hawkins et al., 1991; Emery and Weymouth, 1997; Dennison and Morris, 2002). *C. sakazakii* infections in these age groups raise concerns about the survival and growth characteristics of the pathogen in foods other than powdered and reconstituted infant formula and milk. *C. sakazakii* has been isolated from a wide range of foods and beverages (Table 1), thereby posing some level of safety risk to the consumer. Information about how the behavior of *C. sakazakii* on these foods is affected by conditions to which they are exposed would be meaningful when developing strategies and interventions for its control.

Summarized here is a series of experiments conducted in our laboratories. No attempt is made to review the numerous excellent studies reported internationally. The text evolved from a presentation at an International Meeting on *Cronobacter* (*E. sakazakii*) in Dublin, Ireland, 22–23 January 2009 at which we were invited to give an overview of our *Cronobacter* research. Objectives of our work were to better define the survival and growth characteristics of *C. sakazakii* upon exposure to environments and conditions mimicking those imposed by processes and practices followed in commercial channels

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Table 1

Some foods and beverages other than powdered infant formula, powdered milk, and water from which *C. sakazakii* has been isolated.

Food/beverage	Reference
Animal origin	
Camel	Al-Dughan and Yassiem (2001)
Cheese	Leclercq et al. (2002), Roig-Sagués et al. (2002), Iversen and Forsythe (2004), Ogier et al. (2004), Liu et al. (2005), Chaves-Lopez et al. (2006), El-Sharoud et al. (2008)
Eggs	Cabassi et al. (2004), Musgrove et al. (2004)
Fish and fresh products	Mensah et al. (2002), Nketsia-Tabiri et al. (2003), Miranda et al. (2003), Liu et al. (2005)
Meat products	Kimura et al. (1999), Leclercq et al. (2002)
Milk	Choi et al. (1999), Jayaro and Wang (1999), Lafarge et al. (2004), Ogier et al. (2004)
Pork (dry, raw, cured)	Castano et al. (2001), Molloy et al. (2008)
Poultry	Jimenez et al. (2003)
Sausage	Goulet and Picard (1986), Schalch et al. (1994), Leclercq et al. (2002)
Shellfish	Balebona et al. (1990)
Shrimp	Teuber (2001), Kim et al. (2008b)
Plant origin	
Attikié (fermented cassava product)	Coulin et al. (2006)
Barley (green malt)	Scheepe-Leberkühne and Wagner (1986)
Biscuits	Liu et al. (2005)
Cereal (adult and infant)	Restiano et al. (2006)
Courgette	Gray et al. (2001)
Cowpea paste	Bulgarelli et al. (1988)
Dry ingredients (almonds, coconut powder, pistachio, lentils, sponge mix, soup, beanfeast, vegetable suet)	Iversen and Forsythe (2004)
Dumpling	Leuschner et al. (2004a,b)
Fufu (pounded cassava)	Mensah et al. (2002)
Grains, flour or meal (corn, rice, soy, wheat)	Iversen and Forsythe (2004), Restiano et al. (2006), Shaker et al. (2007)
Herbs and spices	Iversen and Forsythe (2004), Restiano et al. (2006)
Khamir (fermented sorghum bread)	Gassem (1999)
Laver (red algae)	Jung and Park (2006)
Lettuce	Francis and O'Beirne (1998), Soriano et al. (2001)
Nuts and seeds	Freire and Offord (2002), Iversen et al. (2004a,b,c)
Pea soup powder	Leuschner et al. (2004a,b)
Rice	Cottyn et al. (2001), Jung and Park (2006)
Seed sprouts	Geiges et al. (1990), Robertson et al. (2002), Cruz et al. (2004), Kim et al. (2009)
Sobia (fermented beverage)	Gassem (2002)
Sous (licorice beverage)	Nassereddin and Yamani (2005)
Soy protein, lentils	Iversen and Forsythe (2004)
Spices	Restiano et al. (2006)
Sweets	Liu et al. (2005)
Tea	Tamura et al. (1995), Zhao et al. (1997)
Tempé (fermented soybean)	Denter and Bisping (1994)
Tofu	Fouad and Hegeman (1993), No et al. (2002)
Tomato	Mensah et al. (2002), Jung and Park (2006)
Vegetables	Osterblad et al. (1999), Leclercq et al. (2002)
Vegetables (mixed salad)	Galli et al. (1990), Geiges et al. (1990), Ottaviani et al. (1992), Lack et al. (1999), Weiss et al. (2005)

and in food storage and preparation areas in hospitals, day-care centers, and the home.

2. Recovery of stressed cells

Several differential and selective media have been developed for detecting or enumerating *C. sakazakii* in clinical, food, and environmental samples (Hsing-Chen and Wu, 1992; Iversen et al., 2004b; Leuschner et al., 2004a,b; Oh and Kang, 2004). While these media are promising for recovering the pathogen from various sources, their suitabilities for supporting repair of stressed or injured cells and colony development by these cells were not compared. *C. sakazakii* is

known to undergo injury upon exposure to chemical and physical stresses (Breeuwer et al., 2003; Barron and Forsythe, 2007; Al-Holy et al., 2008; Osaili et al., 2008a,b; Shaker et al., 2008).

We compared the suitability of agar media to resuscitate and support colony development by healthy and heat-, freeze-, acid-, alkaline-, and desiccation-stressed cells of *C. sakazakii* (Gurtler and Beuchat, 2005). Cells of *C. sakazakii* exposed to heat (55 °C for 5 min), freezing (−20 °C for 24 h, thawed, frozen again at −20 °C for 2 h, thawed), acidic pH (3.6 adjusted with lactic acid), alkaline pH (11.3 adjusted with sodium hydroxide), and desiccation in powdered infant formula (a_w 0.25, 25 °C for 30 days) were surface plated on eight test media. Tryptic soy agar supplemented with pyruvate, used as a control, supported colony development by the highest number of stressed cells (Table 2). Overall, Leuschner et al. (2004a) agar performed best for recovering stressed *C. sakazakii*. Test media possessing greater selective characteristics clearly inhibited resuscitation and colony development. The poor performance by Enterobacteriaceae enrichment (EE) agar raises concern about the ability of EE broth, which has been used as an enrichment broth in research and testing laboratories, to provide conditions necessary to recover stressed *C. sakazakii*.

3. Survival and growth in infant formulas and in powdered milk

3.1. Survival in powdered infant formula

Differences in composition of infant formulas, coupled with differences in a_w and storage temperature, are likely to affect the survival of *C. sakazakii* in powdered infant formula and other foods. The pathogen is known to survive for at least two years in powdered infant formula at low a_w (Edelson-Mammel et al., 2005; Barron and Forsythe, 2007). We undertook a study to determine the effects of a_w and storage temperature on the survival characteristics of the pathogen in four commercially manufactured milk-based and two soybean-based powdered infant formulas (Gurtler and Beuchat, 2007c). A mixture of ten strains of *C. sakazakii* isolated from infected infants (five strains), foods (four strains), and the environment (one strain) was spray-inoculated into powdered milk. The dried inoculum was added to the six infant formulas at three a_w ranges (0.25–0.30, 0.31–0.33, and 0.43–0.50) to give low (log 0.80 CFU/g) and high (log 4.66–4.86 CFU/g) populations. Formulas were stored at 4, 21, and 30 °C for 12 months during which samples were analyzed for the presence (by enrichment) and populations of *C. sakazakii*.

Populations in formulas initially containing a high inoculum decreased significantly over time at all a_w and storage temperature combinations. Shown in Fig. 1 are numbers of *C. sakazakii* recovered from a milk-based formula at a_w 0.26, 0.31, and 0.44. Changes in populations in the other five formulas stored under the same conditions were similar. When examining the effects of a_w of formulas, populations of *C. sakazakii* in five of six formulas at a_w 0.43–0.50 were significantly reduced compared to populations in formulas at a_w 0.25–0.30 when storage was at 4 °C for 6 months. Decreases in populations were greater in formulas stored at 21 and 30 °C than at 4 °C, and greater at 30 °C than at 21 °C. In three of the six formulas (a_w 0.43–0.50) stored for 6 months and five of six formulas stored for 9 months at 21 °C, initially high populations decreased to ≤ 1 log CFU/g. *C. sakazakii* was detectable only by enrichment (detection limit was 1 CFU/10 g) of four of six formulas stored at 30 °C for 3 months. The pathogen was detected by enrichment in only three of six formulas (a_w 0.43–0.50) stored at 30 °C for 6 months.

In powdered infant formulas inoculated with a low population of *C. sakazakii* (0.80 log CFU/g) and stored for 12 months, the pathogen was detected by enrichment of 17 of 18 (94%), 7 of 18 (39%), and 2 of 18 (11%) formula/ a_w combinations stored at 4, 21, and 30 °C, respectively. Survival was favored by low a_w and at low storage temperature. Inactivation was generally unaffected by composition of

Table 2
Populations of control, heat-stressed, freeze-stressed, acid-stressed, and alkaline-stressed cells of a composite of four strains of *C. sakazakii* recovered on nonselective and differential, selective media.

Recovery medium ^a	Population (log CFU/ml) recovered ^b											
	Control cells	R ^c	Heat-stressed cells	R ^c	Freeze-stressed cells	R ^c	Acid-stressed cells	R ^c	Alkaline-stressed cells	R ^c	All stress conditions	R ^d
TSAP	9.23 a		8.54 a		8.08 a		8.45 a		8.75 a		8.51 a	
LBDC	9.04 b	0.19	8.31 b	0.23	8.02 a	0.06	8.21 b	0.24	8.70 a	0.05	8.39 b	0.12
FCA	8.96 bc	0.27	8.02 bc	0.52	7.48 b	0.60	7.22 c	1.23	8.33 b	0.42	7.96 c	0.55
DFI	8.88 bc	0.35	7.66 c	0.88	7.03 b	1.05	6.73 c	1.72	8.25 b	0.50	7.78 d	0.73
OK	8.85 c	0.38	7.87 c	0.67	7.29 b	0.79	6.89 c	1.56	8.26 b	0.49	7.85 cd	0.66
VRBG	8.79 cd	0.44	7.51 c	1.03	7.24 b	0.84	6.86 c	1.59	8.29 b	0.46	7.80 cd	0.71
EE	8.42 d	0.81	7.37 c	1.17	6.66 b	1.42	6.56 c	1.89	7.82 c	0.93	7.39 e	1.12

From Gurtler and Beuchat (2005), reproduced with permission from the American Society for Microbiology, Washington, D.C., U.S.A.

^a Tryptic soy agar supplemented with 0.1% pyruvate (TSAP); Leuschner, Baird, Donald, and Cox (LBDC) agar (a differential, nonselective medium) (Leuschner et al., 2004a,b); fecal coliform agar (FCA) (Hsing-Chen and Wu, 1992); Druggan–Forsythe–Iversen (DFI) medium (Iversen et al., 2004a); Oh and Kang (OK) agar (Oh and Kang, 2004); violet red bile glucose (VRBG) agar; and *Enterobacteriaceae* enrichment (EE) agar (EE broth supplemented with 1.5% agar).

^b Within each column, mean values that are not followed by the same letter are significantly different ($P \leq 0.05$).

^c Within control or stress condition, reduction in the number of cells recovered on differential, selective media compared to the number of cells recovered on TSAP.

^d Within a composite of all stress conditions, reduction in the number of cells recovered on differential, selective media compared to the number recovered on TSAP.

the formulas. These studies clearly indicate that *C. sakazakii* can survive in powdered infant formula for long periods of time. Survival is influenced by a_w and temperature but not formula composition.

Survival characteristics of one clinical strain and one environmental strain of *C. sakazakii* in a milk-based infant formula and a soybean-based formula at a high a_w range (0.43–0.86) during storage at 4, 21, and 30 °C for up to 24 weeks were studied (Gurtler and Beuchat, 2007c). Fig. 2 shows inactivation curves for the clinical strain in milk-based formula at a_w 0.52–0.86. With the exception of the environmental strain in soybean-based formula stored at 4 °C, initial populations of *C. sakazakii* (4.98–7.07 log CFU/g) in powdered infant formulas initially at this high a_w decreased significantly at all a_w and temperature combinations during a 24-week storage period. At a given storage time, populations were often significantly lower in formulas at higher a_w and at higher storage temperatures. Except for the milk-based formula (a_w 0.75–0.86) inoculated with the clinical strain, regardless of the a_w , *C. sakazakii* was detected by surface plating formulas stored for 24 weeks at 4 °C. In the soybean-based formula (a_w 0.65–0.81) and in milk-based formula (a_w 0.57) stored for 24 weeks at 30 °C, the environmental strain was detected only by

enrichment. The clinical strain was not detected by enrichment of formulas stored at 21 or 30 °C for 24 weeks. Regardless of formula composition or storage temperature, death of the two strains was more rapid in formulas in the high a_w range than was the ten-strain mixture in formulas at lower a_w (0.25–0.50).

Differences in survival characteristics of the two strains of *C. sakazakii* in formulas were attributed, in part, to differences in phenotypic characteristics (Gurtler and Beuchat, 2007c). The clinical strain produced mucoidal colonies on violet red bile glucose agar supplemented with pyruvate while the environmental strain formed crinkled, matte colonies with a tough, rubbery texture. The ability of the environmental strain to survive longer than the clinical strain in formulas suggests that the production of mucoidal material by *C. sakazakii* does not necessarily correlate with protection against death caused by desiccation.

3.2. Survival in powdered milk

Populations of *C. sakazakii* in the powdered milk inoculum used in the studies on survival of the pathogen in powdered infant formula

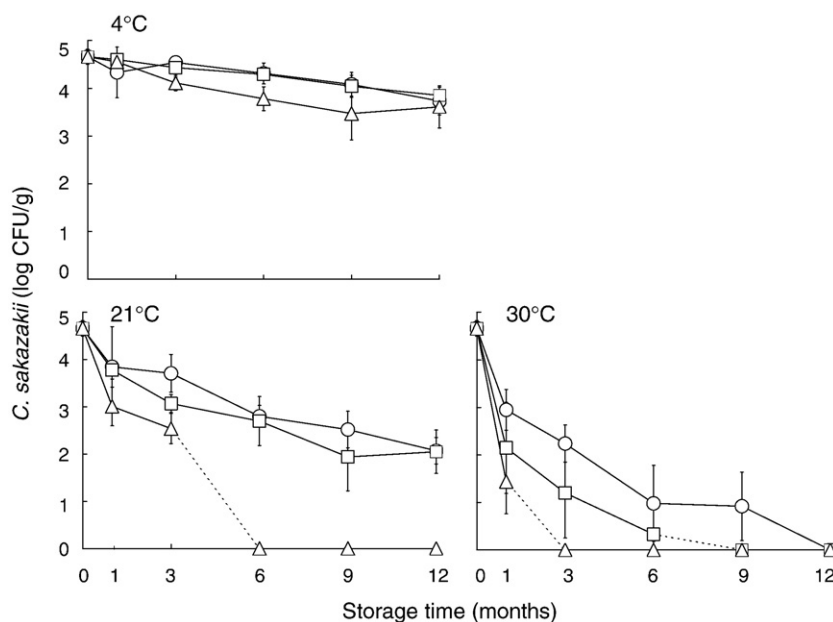


Fig. 1. Number of *C. sakazakii* recovered from a milk-based powdered infant formula as affected by initial a_w 0.26 (○), 0.31 (□), and 0.44 (△), and storage at 4, 21, and 30 °C. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that *C. sakazakii* was detected in one or more of three replicate samples. From Gurtler and Beuchat (2007c), reprinted with permission of Journal of Food Protection, copyright held by the International Association for Food Protection, Des Moines, Iowa, U.S.A.

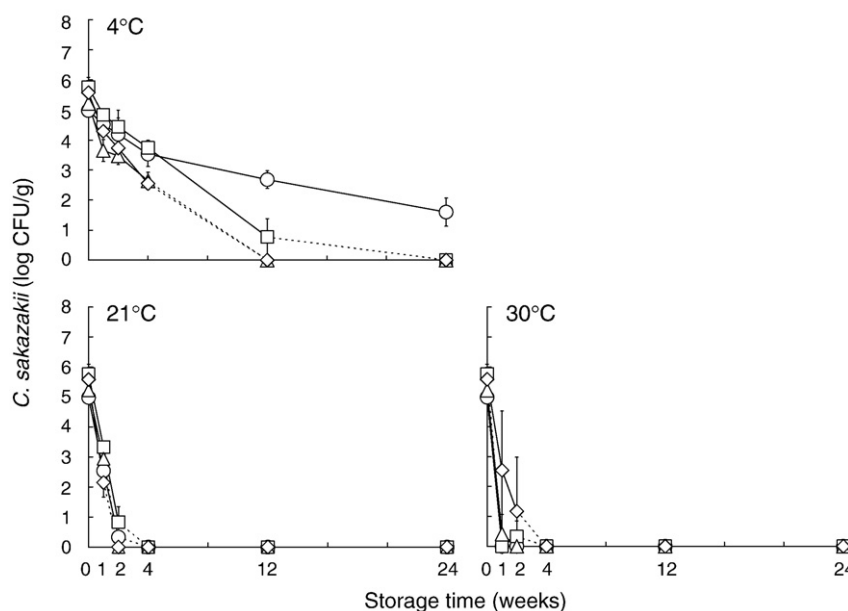


Fig. 2. Number of a clinical strain of *C. sakazakii* recovered from a milk-based powdered infant formula as affected by initial a_w 0.52 (○), 0.75 (□), 0.81 (△), and 0.86 (◇) and storage at 4, 21, and 30 °C. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that *C. sakazakii* was detected in one or more of three replicate samples. From Gurtler and Beuchat (2007c), reprinted with permission of Journal of Food Protection, copyright held by the International Association for Food Protection, Des Moines, Iowa, U.S.A.

described above were monitored over a 12-month period. The a_w of the powdered milk was 0.28 and the storage temperature was at 21 °C for 7 days, followed by storage at 3 °C for 24 days. An initial population of 6.82 log CFU/g decreased significantly but only by about 0.5 and 1 log CFU/g after 9 and 12 months, respectively (Gurtler and Beuchat, 2007c).

3.3. Growth in reconstituted infant formula

Although the ability of *C. sakazakii* to grow in reconstituted powdered infant formula has been reported by others (Nazarowec-White and Farber, 1997b; Iversen et al., 2004b; Kandhai et al., 2006; Lenati et al., 2008), its behavior as affected by the composition of the formula and temperature after reconstituting has not been fully defined. We did a study in which the same ten-strain mixture of *C. sakazakii* used in studies focused on determining the survival characteristics in powdered infant formula (Gurtler and Beuchat, 2007c) were used to inoculate the same six milk-based and soybean-based formulas after reconstituting with water (Gurtler and Beuchat, 2007a). Initial inocula of 0.02 and 0.53 CFU/ml (approximately 13 and 409 CFU/100 g of powdered formula, respectively) were tested. Populations of *C. sakazakii* in reconstituted formulas stored at 4, 12, 21, and 30 °C for up to 72 h were determined.

The pathogen did not grow in reconstituted formulas stored at 4 °C but it was detected by enrichment in all six formulas 72 h after reconstitution. The low inoculum (0.02 CFU/ml) increased to populations exceeding 1 log CFU/ml of various formulas stored for 48, 12, and 8 h at 12, 21, and 30 °C, respectively. The pathogen grew from an initial higher population of 0.53 CFU/ml to populations exceeding 1 log CFU/ml of reconstituted formula held for 24 and 8 h at 12 and 21 °C, respectively, and to populations of 2.55–3.14 log CFU/ml when stored for 8 h at 30 °C. Growth was not greatly affected by the composition of the formula. Shown in Fig. 3 are growth curves of *C. sakazakii* in a reconstituted milk-based formula. Behavior of the pathogen in the other five formulas was similar. Decreases in populations after various storage times are attributed in part to decreases in pH of the reconstituted formulas. An initial pH of 6.7–7.1 decreased to 4.3–5.0 in formulas inoculated with either 0.02 or 0.53 CFU/ml and stored at 30 °C for 72 h, thereby inhibiting growth and causing lethality to a

portion of cells. To minimize the potential for growth of *C. sakazakii* in reconstituted formula, it was recommended that the time held at room temperature be less than 4 h. To prevent growth, reconstituted formula should be stored at ≤ 4 °C.

3.4. Inhibition of growth by the lactoperoxidase system

The lactoperoxidase system (LPOS) consists of three major components, viz., lactoperoxidase (LPO), thiocyanate (SCN^-), and hydrogen peroxide, which act on cytoplasmic membranes of microbial cells by oxidizing sulfhydryl groups on enzymes and proteins. Antimicrobial activity of LPOS against several foodborne pathogens and spoilage microorganisms has been described (Stopforth et al., 2005). The use of LPOS to control microorganisms in infant milk formula has been reported (Banks and Board, 1985; Ernschaw et al., 1990). We studied the effectiveness of LPOS as an inhibitor of *C. sakazakii* in a milk-based powdered infant formula reconstituted with water (Gurtler and Beuchat, 2007b). Initial populations of 0.04 CFU/ml grew to 2.40–2.74 log CFU/ml in reconstituted formula stored for 8 h at 30 or 37 °C and to 0.6 log CFU/ml in formula stored for 12 h at 21 °C. In contrast, the pathogen was not detected (<1 CFU/220 ml) by enrichment of formula supplemented with 10–30 µg/ml LPO and stored for 24 h at 37 °C (Table 3). The inhibitory activity of LPOS was also evident when inoculated formula was held at 21 or 30 °C. These results show that LPOS has the potential to either inactivate *C. sakazakii* or prevent its growth in reconstituted powdered infant formula. It is cautioned, however, that this intervention should not be used in lieu of good manufacturing and hygienic practices.

4. Survival and growth in infant cereal

4.1. Survival in dry infant cereals

Milled, reconstituted cereals are common weaning foods for infants at the age of 4–6 months. The immune systems in these infants is not fully developed. We and others have described conditions affecting the survival of *C. sakazakii* in powdered and reconstituted infant formula but not dry or reconstituted infant cereal. Recognizing that other foodborne pathogens, e.g., enterohemorrhagic

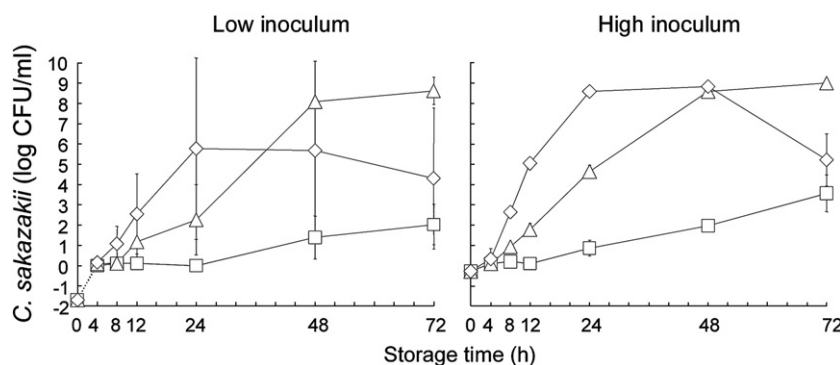


Fig. 3. Number of *C. sakazakii* recovered from a reconstituted milk-based infant formula inoculated at low (0.02 CFU/ml) or high (0.53 CFU/ml) populations of the pathogen and stored at 12 (□), 21 (Δ), or 30 °C (◇) for up to 72 h. Bars indicate standard deviations. Detection limit was 1 CFU/ml (0 log CFU/ml). From Gurtler and Beuchat (2007a), reprinted with permission of Journal of Food Protection, copyright held by the International Association for Food Protection, Des Moines, Iowa, U.S.A.

Escherichia coli O157:H7 (Deng et al., 1998) and toxigenic *Bacillus cereus* (Jaquette and Beuchat, 1998), can survive for long periods of time in dry cereal and grow well in reconstituted cereal, we undertook studies to determine the survival characteristics of *C. sakazakii* in dry infant rice, barley, mixed grain, and oatmeal cereals as affected by a_w (0.30–0.83) and temperatures (4, 21, and 30 °C) at which they are held during distribution at retail or in hospital, day-care center, and home settings (Lin and Beuchat, 2007b). Populations of *C. sakazakii* decreased in all inoculated cereals held for 24 weeks at 4, 21, or 30 °C. Fig. 4 shows inactivation curves for the pathogen initially at a high population (4.5–5.8 log CFU/g) in cereals stored at 21 °C for 24 weeks. Increases in a_w of cereals and storage temperature accelerated the rate of death. At an initial low population of 0.31 log CFU/g, *C. sakazakii* survived in rice cereal (a_w 0.30–0.69) for up to 12 months at all storage temperatures. Survival was not markedly affected by the type of cereal (a_w 0.63–0.83) or storage temperature. This study showed that *C. sakazakii* can survive for up to 12 months in infant cereals having a wide range of a_w when cereals are stored under conditions simulating those to which they may be exposed during distribution, at retail, and in the home.

4.2. Survival and growth in reconstituted infant cereal

We conducted two studies to determine the survival and growth characteristics of *C. sakazakii* in reconstituted infant cereals. The first study focused on rice cereal reconstituted with water, apple juice not containing added preservatives, milk or liquid infant formula (Richards et al., 2005). A ten-strain mixture of *C. sakazakii* was inoculated into reconstituted cereal at populations of 0.27, 0.93, and 9.3 CFU/ml and stored at 4, 12, 21, or 30 °C. Growth did not occur within 72 h in cereal reconstituted with apple juice, regardless of storage temperature, or in cereal reconstituted with water, milk, or formula and stored at 4 °C. The inability of the pathogen to grow in rice cereal reconstituted with apple juice was attributed to acidic pH (4.29). *C. sakazakii* grew in rice cereal reconstituted with water, milk, or liquid infant formula and stored at 12, 21, or 30 °C. This study revealed that *C. sakazakii* initially at very low populations can rapidly grow in reconstituted infant rice cereal.

A second series of studies was done to compare survival and growth characteristics of *C. sakazakii* in reconstituted rice cereal, rice with mixed fruit cereal, and oatmeal cereal (Lin and Beuchat, 2007a). Infant cereals were reconstituted with water, milk, or apple juice, inoculated with a ten-strain mixture of *C. sakazakii* at populations of 0.005 and 0.52 CFU/ml, and stored at 4, 12, 21, or 30 °C. Populations of the pathogen were monitored over a 72-h period. Growth did not occur in cereals held at 4 °C or in cereals reconstituted with apple juice and stored at 12 °C. This confirmed observations from the first study on the behavior of different strains of *C. sakazakii* in reconstituted rice cereal. The pathogen was detected (≥ 1 CFU/ml) in the three cereals

reconstituted with water or milk and stored for 24, 8, and 4 h at 12, 21, and 30 °C, respectively. Rapid growth subsequently occurred in cereals stored at 21 or 30 °C (Table 4). Survival and growth were not markedly affected by the composition of cereals. *C. sakazakii* grew at 21 and 30 °C within 2 days in rice cereal reconstituted with apple juice, then decreased to undetectable levels (<1 CFU/10 ml) in cereal stored at 21 °C for 5 days or 30 °C for 4 days. Initially at 7.32 log CFU/ml, *C. sakazakii* was detected in rice cereal reconstituted with apple juice and stored at 4 °C for 50 days. Results of this study provide further evidence that very low numbers of *C. sakazakii* can grow to large populations in reconstituted infant cereals. Rates of inactivation or growth are affected by the type of liquid used to reconstitute cereals and the temperature at which they are stored after reconstitution.

5. Biofilm formation and resistance to disinfectants

5.1. Attachment and formation of biofilm

Attachment of bacteria and fungi to surfaces may be followed by the production of exopolysaccharide and biofilm formation (Kumar and Anand, 1998). *C. sakazakii* has been reported to be able to attach to abiotic materials such as silicon, latex, polycarbonate, stainless steel, glass, and polyvinyl chloride and form biofilms (Iversen et al., 2004b; Lehner et al., 2005; Grimm et al., 2008). We examined conditions affecting attachment of and biofilm formation by *C. sakazakii* on stainless steel and enteral feeding tubes (Kim et al., 2006a). Cells of

Table 3

Presence of *C. sakazakii* detected by enrichment of reconstituted milk-based infant formula inoculated with a low population (0.04 CFU/ml), treated with LPOS, and incubated for up to 24 h at 21, 30, or 37 °C.

Storage temp. (°C)	Lactoperoxidase concentration (μg/ml)	Number of positive samples ^a			
		4 h	8 h	12 h	24 h
21	0	3	3	3	3
	10	0	0	0	1
	20	0	0	0	2
	30	0	0	0	1
30	0	3	3	3	3
	10	0	0	2	1
	20	0	0	1	1
	30	0	1	1	0
37	0	3	3	3	3
	10	0	0	0	0
	20	0	0	0	0
	30	0	0	0	0

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^a Numbers indicate samples positive for *C. sakazakii* out of three tested (three replicate experiments). The initial population was 0.04 CFU/ml and the detection limit was 1 CFU/10 ml at 4, 8, and 12 h and 1 CFU/220 ml at 24 h.

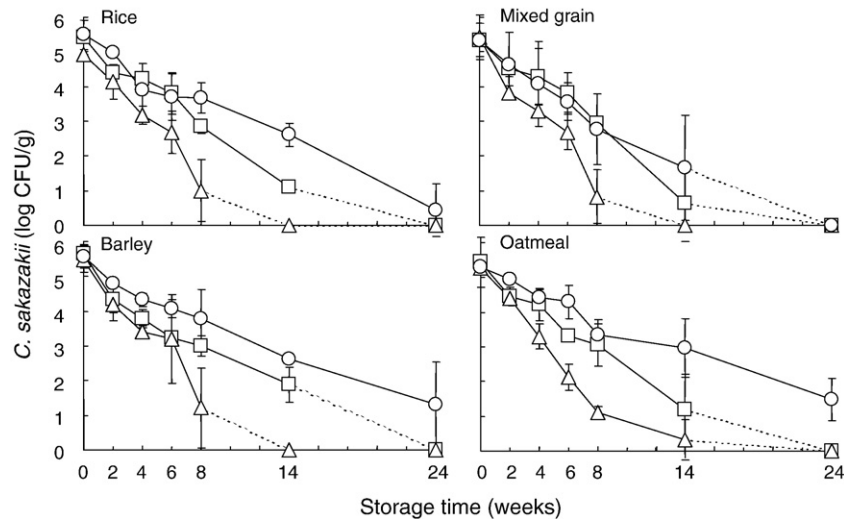


Fig. 4. Number of *C. sakazakii* recovered from dry infant rice, barley, mixed grain, and oatmeal cereals initially at aw 0.63–0.66 (\circ), 0.76 (\square), and 0.82–0.83 (Δ) and stored at 21 °C for up to 24 weeks. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that *C. sakazakii* was detected in one or more of three replicate samples. From Lin and Beuchat (2007b), reprinted with permission of Journal of Food Protection, copyright held by the International Association for Food Protection, Des Moines, Iowa, U.S.A.

five strains grown in tryptic soy broth, infant formula broth, and lettuce juice were studied for their ability to attach to surfaces of stainless steel and feeding tubes. Higher numbers of cells attached at 25 °C than at 12 °C. Stainless steel coupons and enteral feeding tubes were immersed for 24 h at 4 °C in suspensions (7 log CFU/ml) to facilitate the attachment of 5.33–5.51 and 5.03–5.12 log CFU/cm², respectively, before immersing in tryptic soy broth, infant formula broth, or lettuce juice at 12 or 25 °C for up to 20 days. Biofilms were not formed on stainless steel or tubes at 12 °C, regardless of the immersion liquid, or at 25 °C when immersed in sterile tryptic soy broth or lettuce juice. However, the pathogen did form biofilms on surfaces of both materials immersed in infant formula broth at 25 °C, indicating that type and availability of nutrients plays a role in processes leading to biofilm formation. These findings reinforce the

importance of preventing contamination of contact surfaces in areas where powdered infant formulas are reconstituted or fed to infants.

Protection of *C. sakazakii* by organic matrices upon drying on the surface of stainless steel coupons was studied (Kim et al., 2008a,b). The number of *C. sakazakii* per coupon (initial population of 7.4–8.6 log CFU/coupon) decreased significantly at 4, 25, and 37 °C within 10, 3, and 1 day(s), respectively, at 43% relative humidity, but remained viable for 60 days (Fig. 5). Reductions were significantly greater when cells had been suspended in water rather than infant formula before drying on the surface of stainless steel. Reductions were greater at 37 °C, compared to 4

Table 4

Estimated generation times for *C. sakazakii* in infant cereals reconstituted with water or milk^a.

Reconstitution liquid	Incubation Temp. (°C)	Period (h) ^b	Cereal	Generation time
Water	12	24–72	Rice	2.73 h
			Oatmeal	3.62 h
			Rice with mixed fruit	5.05 h
	21	8–24	Rice	54.2 min
			Oatmeal	66.6 min
			Rice with mixed fruit	63.4 min
	30	4–12	Rice	33.3 min
			Oatmeal	31.8 min
			Rice with mixed fruit	30.2 min
Milk	12	24–72	Rice	4.90 h
			Oatmeal	4.75 h
			Rice with mixed fruit	5.16 h
	21	8–24	Rice	62.7 min
			Oatmeal	57.2 min
			Rice with mixed fruit	54.8 min
	30	4–12	Rice	32.0 min
			Oatmeal	32.3 min
			Rice with mixed fruit	30.4 min

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^a Initial population was 0.52 CFU/ml.

^b Incubation period indicates segment of growth curve used to calculate generation time.

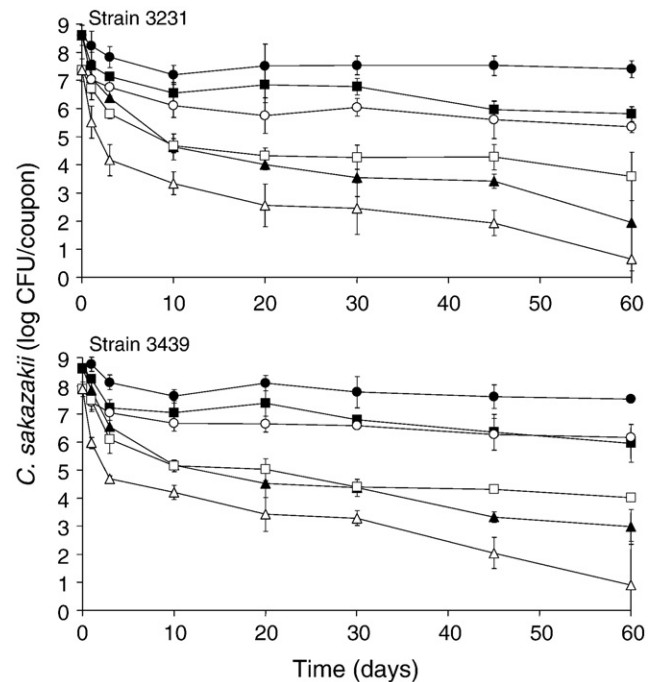


Fig. 5. Number of *C. sakazakii* recovered from stainless steel coupons on which cell suspensions in distilled water (open symbols) and infant formula (closed symbols) were applied and dried. Coupons were stored at 4 °C (circles), 25 °C (squares), and 37 °C (triangles) under a relative humidity of 43% for up to 60 days. Error bar indicate standard deviations. From Kim et al. (2008a), reprinted with permission of Journal of Food Protection, copyright held by the International Association for Food Protection, Des Moines, Iowa, U.S.A.

or 25 °C, regardless of the type of cell carrier. In the same study, biofilm formation by *C. sakazakii* on stainless steel coupons immersed in a minimal nutrient medium or infant formula at 25 °C, followed by exposure of coupons to relative humidities of 23, 43, 68, 85, and 100% at 25 °C for up to 42 days, was investigated. The overall order of survival as affected by relative humidity was $100 > 23 = 43 = 68 > 85$, regardless of the medium in which biofilm had been formed. These studies show that cells of *C. sakazakii* in biofilms formed in infant formula as a nutrient source are protected against lethality when exposed to reduced-moisture conditions. These observations are of value when predicting survival characteristics of the pathogen on stainless steel surfaces in processing and preparation kitchen environments.

5.2. Resistance to disinfectants

The presence of *C. sakazakii* on the surface of equipment and utensils used in infant formula preparation has been documented in clinical settings where neonatal infections have occurred (Clark et al., 1990; Bar-Oz et al., 2001). An assessment of temperature conditions in neonatal care units relative to survival and growth of *C. sakazakii* in infant formula has been done (Rosset et al., 2007). Although disinfection of surfaces in formula preparation areas in hospitals, food service kitchens, and day-care centers is routinely carried out, the efficacy of various disinfectants in killing *C. sakazakii* on these surfaces has received meager research attention. We undertook an investigation to determine the effectiveness of disinfectants in killing the pathogen in suspension, dried on the surface of stainless steel, and embedded in biofilm on stainless steel (Kim et al., 2007). Thirteen quaternary ammonium and phenolic disinfectants commonly used in infant formula preparation areas were evaluated. Depending on the disinfectant, amount and type of organic matrix surrounding cells, and exposure time, *C. sakazakii* showed various levels of resistance. Populations of planktonic cells suspended in water (7.22–7.40 log CFU/ml) decreased to undetectable levels (<0.30 log CFU/ml) within 1–5 min upon treatment with disinfectants, while numbers of cells in reconstituted infant formula were reduced by only 0.02–3.69 log

CFU/ml after treatment for 10 min. Overall, the ineffectiveness of disinfectants in killing *C. sakazakii* in biofilm on stainless steel was most evident. Cells dried on stainless steel were less resistant to disinfectants, and planktonic cells were least resistant.

Protection of *C. sakazakii* in biofilms against disinfectants is not an unexpected finding. Diminished bactericidal activity of disinfectants and sanitizers against cells in biofilms has been described by others (deBeer et al., 1994; Mah and O'Toole, 2001). The ability of *C. sakazakii* to produce exopolysaccharides, which enhances biofilm formation (Scheepe-Leberkühne and Wagner, 1986; Iversen et al., 2004b; Lehner et al., 2005; Grimm et al., 2008; Kim et al., 2008a,b), coupled with its increased resistance to treatment with disinfectants when embedded in organic matrices emphasizes the importance of proper cleaning of surfaces soiled by infant formula. Inadequate cleaning may compromise the lethality of disinfectants routinely used in formula preparation and feeding area in hospitals, day-care centers, and homes.

6. Survival and growth on fresh-cut fruits and vegetables, and efficacy of sanitizers

6.1. Survival and growth on produce and in unpasteurized juice

Among the ready-to-eat foods from which *C. sakazakii* has been isolated are lettuce (Soriano et al., 2001), seed sprouts (Kim et al., 2009), and other vegetables (Geiges et al., 1990; Ottaviani et al., 1992; Leclercq et al., 2002; Weiss et al., 2005). The incidence of foodborne diseases associated with consumption of fresh produce has increased in recent years. The ability of *C. sakazakii* to grow at temperatures as low as 5.5 °C (Nazarowec-White and Farber, 1997b) raises concern about its ability to grow on refrigerated fresh-cut produce, thereby increasing the risk of infections in immunocompromised consumers.

We did a study to determine the survival and growth characteristics of *C. sakazakii* on fresh-cut produce and in unpasteurized fruit and vegetable juices (Kim and Beuchat, 2005). Fresh-cut apples, cantaloupes, strawberries, watermelon, cabbage, carrots, cucumbers, iceberg lettuce, and tomatoes were inoculated (2–3 log CFU/g) with a

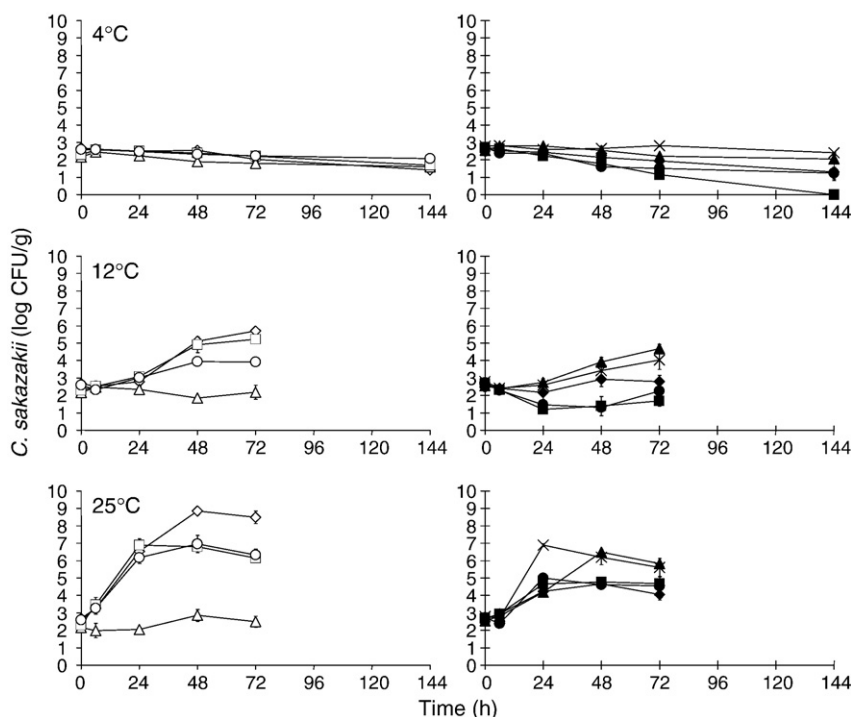


Fig. 6. Number of *C. sakazakii* recovered from fresh-cut apples (○), cantaloupe (□), strawberries (Δ), watermelon (◇), cabbage (●), carrot (■), cucumber (▲), lettuce (◆), and tomato (×) stored at 4, 12, or 25 °C for up to 6 days. From Kim and Beuchat (2005), reprinted with permission of Journal of Food Protection, copyright held by the International Association for Food Protection, Des Moines, Iowa, U.S.A.

five-strain mixture of *C. sakazakii*. Produce was stored at 4, 12, or 25 °C for up to 6 days, during which populations of *C. sakazakii* were monitored. At 4 °C, the number of *C. sakazakii* on produce either did not change or gradually decreased (Fig. 6). With the exceptions of strawberries, cabbage, and carrot stored at 12 °C and strawberries stored at 25 °C, produce supported the growth of *C. sakazakii*. Populations ranging from 4.2–8.9 log CFU/g were reached in produce other than strawberries stored at 25 °C.

C. sakazakii survives but does not grow on the surface of uncut apples, cantaloupes, strawberries, and tomatoes stored for up to 28 days at 4, 12, or 25 °C (Kim et al., 2006b). The rate of death depends on the temperature; the pathogen lost viability more rapidly at 25 °C than at 4 or 12 °C. Regardless of the type of produce, initial populations of 8.60–8.78 log CFU/produce were not reduced by more than 4.03 log CFU/produce during the 28-day storage period. These observations emphasize the need to assess safety risks for uncut as well as fresh-cut produce.

Studies were also done to determine survival and growth characteristics of *C. sakazakii* in unpasteurized fruit and vegetable juice (Kim and Beuchat, 2005). Initial populations of 1.2–1.6 log CFU/ml of cantaloupe, lettuce, and tomato juice decreased slightly during storage of the produce for 7 days at 4 °C but only 0.3 and 0.2 log CFU/ml were detected in strawberry juice and cabbage juice, respectively. The pathogen grew in cantaloupe juice and watermelon juice, but not in the other juices, at 12 °C. All fruit and vegetable juices except apple, strawberry, and cabbage juice supported growth at 25 °C. The highest populations were detected in watermelon juice (8.1 log CFU/ml) and carrot juice (7.3 log CFU/ml) stored at 30 h.

Results of these studies clearly show that *C. sakazakii* can grow to high populations on some types of fresh-cut produce and in fruit and vegetable juices. Exposure of produce and juices that may be contaminated with *C. sakazakii* to temperature abuse during post-harvest handling in retail, food service, and home settings favors proliferation of the pathogen and possibly an increased risk of infection to immunocompromised consumers.

6.2. Efficacy of produce sanitizers

Observations on the ability of *C. sakazakii* to survive and grow on fresh produce and in juice led to experiments designed to determine the efficacy of produce sanitizers. Chlorine, chlorine dioxide, and a peroxyacetic acid-based sanitizer (Tsunami 200®) were evaluated for their effectiveness in killing *C. sakazakii* on apples, iceberg lettuce, and tomatoes (Kim et al., 2006b). At 50 µg/ml, chlorine and chlorine dioxide were equivalent in killing *C. sakazakii* on apples. Treatment with 10 µg/ml chlorine dioxide or 40 µg/ml Tsunami 200 for 1 min caused reductions of 3.38 log CFU/apple and ≥4.00 log CFU/apple, respectively, compared to the number remaining on apples after washing with water. Similar reductions on tomatoes were achieved. Chlorine was less effective in killing *C. sakazakii* on lettuce than on apples or tomatoes. Treatment of lettuce with Tsunami 200 caused a reduction of ≥5.31 log CFU/sample (three 9×9 cm pieces of leaves). Overall, treatment of apples, tomatoes, and lettuce with chlorine, chlorine dioxide, and Tsunami resulted in significant reductions in *C. sakazakii*. The pathogen does not appear to be more resistant to these sanitizers than other enteric bacterial pathogens known to cause outbreaks of infections associated with produce (Beuchat, 2000).

7. Conclusions

C. sakazakii can be found in a wide range of foods and beverages, many of which are not subjected to treatments or processes that will inactivate the pathogen. Its ability to survive and grow in these products raises concern about safety risks not only to neonates and infants but also to older immunocompromised consumers. The suitability of infant cereals and some types of fresh fruits and

vegetables to support luxuriant growth of *C. sakazakii* is of particular concern. The ability of the pathogen to produce biofilms, coupled with its resistance to sanitizers and disinfectants when present in organic matrices, emphasizes the importance of properly cleaning and sanitizing food preparation areas and utensils and containers used to prepare and serve foods to neonates and others in hospital, day-care center, and home settings.

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